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Identification of Archetype and Rearranged Forms of BK Virus in Leukocytes From Healthy Individuals

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BK virus (BKV) establishes a persistent, asymptomatic infection in more than 80% of the human population, and in immunocompromised individuals BKV causes acute urinary tract infections. Two forms of BKV, archetype and rearranged, have been recovered previously from urine samples. The rearranged form is believed to have emerged from the archetype form by the deletion and duplication of sequences within the transcriptional control region (TCR). We have PCR-amplified unique rearranged forms of the BKV TCR from peripheral blood leukocytes (PBLs) of healthy donors. By employing archetype-specific PCR primers, the archetype BKV TCR was also detected in PBLs. These findings are consistent with the hypotheses that PBLs transport BKV to different sites within the body and play a role in the TCR rearrangement process. BKV sequences were detected in 21 of 38 BKV-seropositive and 1 of 2 BKV-seronegative individuals. Because of recent suggestions that SV40 may circulate in the human population, the donors' sera were also examined for the presence of specific anti-SV40 antibodies. A high antibody titer to SV40 was detected in only 1 of the 40 donor specimens. This study is the first to characterize multiple BKV TCR variants in PBLs from healthy people and to correlate PCR and serological methods used to detect BKV infections. ***J. Med. Virol. 60:353–362, 2000.***

KEY WORDS: BKV variants; leukocytes; PCR; ELISA; transcriptional control region

INTRODUCTION

The human polyomavirus, BK virus (BKV) establishes a persistent, asymptomatic infection in most people during their childhood [Gardner et al., 1971; Brown et al., 1975; Dei et al., 1982; Arthur et al., 1986; Flægstad et al., 1991; Agostini et al., 1995], and viruria is a common occurrence in many of these individuals [Chesters et al., 1983; Arthur and Shah, 1989]. In severely immunocompromised patients, BKV, as an opportunistic pathogen, may cause acute hemorrhagic cystitis, ureteral stenosis or interstitial nephritis [Gardner et al., 1971; Coleman et al., 1978; Gardner et al., 1984; McCance and Gardner, 1987; Mathurm et al., 1997]. BKV has also been implicated in some cases of encephalitis and has been detected in human brain and pancreatic islet tumors [Caputo et al., 1983; Corallini et al., 1987; Dörries et al., 1987].

BKV variants recovered from healthy and immunocompromised individuals [Gardner 1973; Shah et al., 1973; Takemoto et al., 1974; Flægstad et al., 1991, 1988; Markowitz et al., 1991] have been classified on the basis of serological and sequence data. Four BKV serotypes have been identified and are believed to circulate in the population [Jin et al., 1995]. A correlation exists between these four serotypes and the genotypes of individual isolates. An isolate belonging to a specific serogroup exhibits a unique set of nucleotide alterations at several positions within its VP1 gene [Knowles et al., 1989; Jin et al., 1993]. These nucleotide polymorphisms alter the amino acid sequence of the VP1 capsid protein between amino acid positions 61 and 83, a region predicted to comprise a major epitope on the capsid surface [Knowles et al., 1989; Jin et al., 1993]. Hence, these different polymorphic forms of BKV (genotypes/serotypes) elicit specific antibody responses from the host immune system.

A second classification scheme, based upon differences within the viral transcriptional control region (TCR), has been used to classify BKV into archetype and rearranged forms. Archetype BKV, the form de-

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tected in urine and thought to circulate in the human population [Yogo et al., 1990; Flægstad et al., 1991; Yogo et al., 1991], has a TCR structure that lacks extensive duplication of the promoter-enhancer sequences. The rearranged form of BKV may represent a collection of viral variants that arise in an individual after the deletion and duplication of sequences within the archetype TCR [Yogo et al., 1990, 1991; Tominaga et al., 1992; Ault and Stoner 1993]. The precise mechanism and site of this rearrangement are unknown, however a similar process may occur in a second human polyomavirus, JC virus (JCV), where it has been suggested that an archetype form undergoes active replication and TCR rearrangement at the site of primary infection [Newman and Frisque, 1997]. The emergent rearranged variants may then be transported to different tissues where they may become established based on their tissue-tropic properties [Martin et al., 1985; Loeber and Dörries, 1988; Newman and Frisque, 1999].

The transport of BKV and JCV to secondary sites of infection may occur via peripheral blood leukocytes (PBLs), and the genomes of both viruses have been detected in these cells [Houff and Major, 1989; Dörries et al., 1994; Smith et al., 1998]. Two laboratories have reported finding rearranged forms of BKV in leukocytes; Dörries et al. [1994] detected BKV(Dun) and Smith and coworkers [1998] identified a new variant, BKV(Cin). No other attempts have been made to identify the form(s) of BKV in PBLs, and no correlation has been established between antibody titers in serum and the presence of viral DNA in PBLs. In the present study we have characterized multiple BKV TCR variants in DNA from the PBLs of healthy donors, and we have also assessed the presence of anti-BKV antibodies in these individuals' sera.

Recently a number of investigators, employing PCR technology, detected SV40 DNA sequences in several different human tumors, and it was suggested that this monkey polyomavirus might circulate in the human population [Carbone et al., 1994; Lednicky et al., 1995; Carbone et al., 1996; Martini et al., 1996; Carbone et al., 1997; Strickler et al., 1998]. In parallel with our BKV studies, we have used ELISA to determine whether anti-SV40 antibodies are present in serum specimens from 40 volunteer donors.

MATERIALS AND METHODS

Human Blood Samples

Blood samples analyzed in this study were obtained from 40 immunocompetent donors between the ages of 19–60 who were students and employees of The Pennsylvania State University. All donors were Caucasian except donors 4 (Native American) and 6 and 39 (both Asian). Brief medical histories were taken for each donor, and conditions potentially relevant to immune status were noted for 14 individuals including shingles (2), organ transplant (1), meningitis (1) and recurrent cold sores (4), bronchitis (3), otitis (4) and diarrhea (1). Experimental protocols involving human subjects were approved by the University's Institutional Review Board. Specimens were collected by University Health Services staff in heparinized tubes to obtain whole blood (10 mls), or in tubes without an anticoagulant to prepare serum (10 mls). Specimens of whole blood were dispensed in sterile Eppendorf tubes in aliquots of 200 μl. DNA was extracted from one aliquot from each donor using the QIAgen Blood Amp Kit (Qiagen Inc., Chatsworth, CA), and the remaining aliquots were stored at −20°C for future use. DNA extraction was conducted in laminar flow hoods to avoid contamination of the samples with aerosolized BKV DNA. To further ascertain that contamination did not occur during the DNA extraction process, sterile water was subjected to the same protocol as whole blood and then analyzed by PCR for the presence of contaminating viral DNA.

PCR Precautions

The potential for contamination associated with PCR analysis has been discussed at length [Kwok, 1990; Jackson et al., 1991; Prince and Andrus, 1992]. To minimize this risk we adopted several recommended precautions [White et al., 1992; Newman and Frisque, 1997; 1999], such as assembling PCR mixes (containing Taq polymerase, PCR buffer, primers and nucleotide triphosphates) under laminar flow hoods in a room separate from where the template DNA was handled, utilizing single-use aliquots of reagents, treating work surfaces and equipment with 1% hypochlorite and UV light, and using positive displacement pipettes (Gilson, Villers-le-Bel, France). In addition, the DNA extraction method used was chosen because it required a minimum number of handling steps, and it employed closed tubes and spin columns. We also included a minimum of four negative controls in each experiment to assess the possibility that contamination may still have occurred despite our precautions.

To determine the sensitivity of our PCR protocol, positive controls containing 10^0 to 10^6 molecules of recombinant BKV(Dun) DNA as the template DNA source were included in each PCR experiment and detectable signals were observed routinely with 10^0 or 10^1 molecules of input DNA. To avoid the possibility of cross-contamination, positive control reactions were assembled only after tubes containing negative controls and leukocyte DNA samples had been closed.

PCR Amplification and Gel Electrophoresis

Each reaction was conducted in a total volume of 50 μl containing thermophilic buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0], 0.1% v/v Triton X-100) (Gibco BRL, Rockville, MD),1.5 mM $MgCl_2$, 2.5 U of Taq polymerase (PGC Scientific, Gaithersburg, MD), 200 μM each of dATP, dCTP, dGTP, and dTTP (Pharmacia Biotech, Uppsala, Sweden) and 0.5 μM of each oligonucleotide primer (Table I). To increase the sensitivity of the reaction and to reduce nonspecific priming, "hot start PCR" using $MgCl_2$ HotWax beads (Invitrogen Inc, San Diego, CA) was employed. Reactions were subjected to

TABLE I. Oligonucleotides Used for PCR and Sequence Analyses[a]

Primer name	Sequence 5′ to 3′	Nucleotide position
BK#2	GTT CCA GTC CAG GTT TTA CC	341–322[b]
BK#3	AAT CAG GCT GAT GAG CTA CC	4826–4845[b], 4770–4789[c]
BK#4	CCG TCT ACA CTG TCT TCA CCT CTA C	422–398[b]
BK#5	GGC CAT TCC TTG CAG TAC AG	4750–4769[b]
BK#6	GGC CAT TCC TTG CAG TAC A	4694–4712[c]
BK(Arch1)	GGC CAG TTT CCA CTT CCT C	184–166[d]
BK(Arch2)	GTC TGG CTG CTT TCC ACT CC	207–188[d]
CD19.3	CCT CTG TGC CTG GCT GTA	49–66[e]
CD19.4	GGG CAA CAC AAC AAG ACC	562–545[e]
M13 Reverse	CAG GAA ACA GCT ATG ACC	205–222[f]
T7 Promoter	TAA TAC GAC TCA CTA TAG GG	365–346[f]

[a]All primers were used for PCR amplification except the M13 reverse and T7 promoter primers which were used to sequence PCR products.
[b]Nucleotide numbering for BKV(Dun) is from Seif et al., [1979].
[c]Nucleotide numbering for BKV(AS) is from Tavis et al., [1989].
[d]Nucleotide numbering for BKV(WW) is from Rubinstein et al., [1987].
[e]Nucleotide numbering for CD19 is from Tedders and Isaacs [1989].
[f]Nucleotide numbering for pCR™2.1 is from the original TA Cloning Kit Manual (Invitrogen).

40 cycles of amplification in either a Perkin-Elmer Cetus DNA thermal cycler or an MJ Research PTC-200 Peltier thermal cycler as described previously [White et al., 1992; Newman and Frisque, 1997].

To enhance the detection of BKV DNA that might be present at very low levels in the donors' leukocytes, nested PCR (n-PCR) was employed. For this purpose, the BK#4 and BK#5 oligonucleotides (Fig. 1; Table I) were used as outer primers to amplify the target DNA through 40 cycles (annealing temperature of 65°C). One μl of the resulting product was further amplified using inner primers BK#2 and BK#3 through 40 cycles (annealing temperature of 60°C). Nested PCR was also employed to detect archetype BKV DNA in leukocytes using archetype-specific primers: BK#6 and BK(Arch2) were used as outer primers and BK#3 and BK(Arch1) were used as inner primers (Table I). To confirm that the DNA extracted from blood was suitable for PCR analysis, a portion of the CD19 (Cluster of Differentiation 19) gene was amplified using primers CD19.3 and CD19.4 [Stamenkovic and Seed, 1988; Tedder and Issacs, 1989] (Table I). PCR products were visualized on gels containing 2% molecular biology certified agarose, TBE buffer (90 mM Trisbase, 90 mM Boric acid, 2.5 mM Sodium-EDTA) and 0.5 μg/ml ethidium bromide and photographed using an EagleEye™ photographic system (Stratagene, La Jolla, CA).

Cloning and Sequence Analysis of PCR Products

PCR products were cloned into the TA cloning vector pCR™2.1 (Invitrogen) as described in the TA-Cloning Kit manual. Recombinant DNA was isolated from ampicillin-resistant *E. coli* transformants using the Wizard Plus Miniprep Kit (Promega, Madison, WI) and digested with *Eco*RI to identify clones containing an insert of the appropriate size. Selected DNAs were sequenced by the Nucleic Acid Facility at The Pennsylvania State University Biotechnology Institute using an ABI 377 Prism Sequencer (Perkin-Elmer, Oak Brook, IL) and M13 reverse and T7 primers (Table I).

Serum Processing and ELISA

Sera were prepared by allowing blood samples collected without anticoagulants to clot during a one hour incubation period at 4°C. The clotted specimens were centrifuged at 10,000 × *g* for 15 minutes, and the upper serum fraction was collected and stored at –20°C to use as a potential source of anti-BKV and anti-SV40 antibodies. Initially, sera titered by the standard hemagglutination inhibition (HAI) assay [Gardner, 1973] were retested by the ELISA method to demonstrate that BKV titers measured by both assays correlated with one another. Once this correlation was established and the ELISA was found to be more sensitive than HAI (data not shown), ELISA was used to determine the antibody titers for the 40 donor sera.

Four BKV serotypes are known to circulate in the population, therefore serum from different people could contain antibodies directed against one or more of the four antigenically distinct forms of BKV. BKV(Dun) is a member of serogroup I [Jin et al., 1993], and it is recognized by antibodies produced against the other BKV serotypes. Thus, BKV(Dun) virions were used as the antigen source to coat the ELISA plates. Intact BKV(Dun) and SV40(776) virions were suspended in coating buffer (15 mM sodium carbonate, 34 mM sodium bicarbonate [pH 9.6]) and adsorbed to 96-well plates (0.5 μg viral protein/well). After incubating the plates overnight at 4°C, unbound virus was removed by 3 washes with TBST (8.8 mM Tris-HCl, 4.37 mM Trisbase, 150 mM NaCl, 0.00005% v/v Tween20 [pH 8.0]). The plates were then treated with 50 μl of blocking solution (1% bovine serum albumin in TBST) per well and incubated at 37°C for 1 hour. Unbound blocking solution was removed by 3 washes with TBST. Serum was serially diluted two-fold in TBST and added to each well in a final volume of 50 μl/well. Anti-BKV or anti-SV40 antibodies present in the serum were allowed to interact with viral antigens during a 2-hour incubation period at 37°C. After washing away unbound serum with TBST, the plates were treated with 50 μl/well of anti-human immunoglobulin (IgG)-

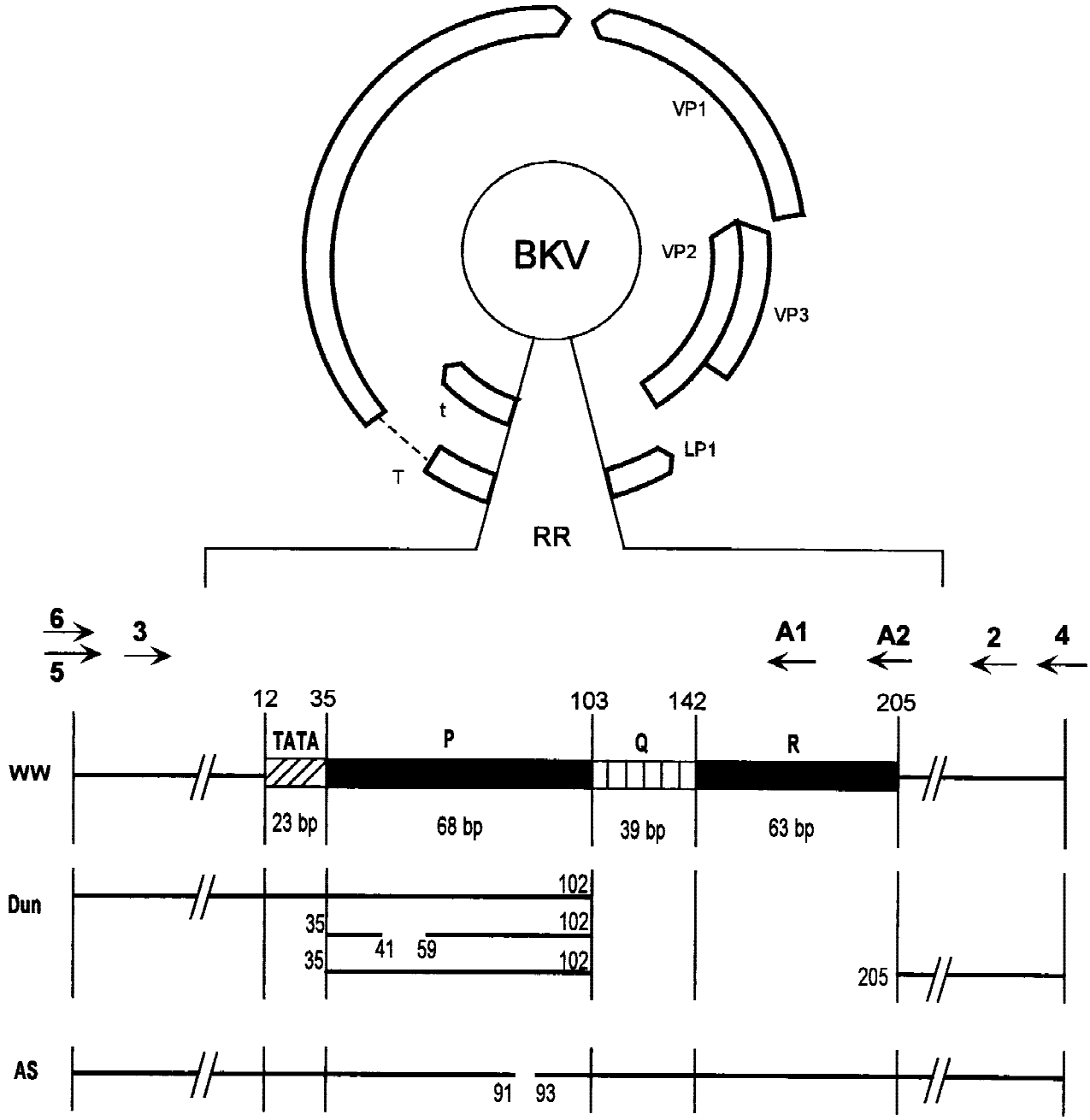

Fig. 1. The BKV genome with the annealing sites of PCR primers within the archetype (WW) and rearranged (Dun and AS) TCRs. The inner circle represents the double-stranded DNA genome (~5100 bp) that encodes at least six viral proteins (outer arrows). The genome is divided into three parts: The early region specifies two regulatory proteins (T and t) translated from alternatively spliced mRNAs. The late region specifies four proteins required for capsid assembly (LP1, VP1, VP2 and VP3). The regulatory region (RR) contains *cis*-acting signals that regulate DNA replication and transcription. The nucleotide numbering used is that of the BKV(WW) genome [Rubinstein et al., 1987]. The BKV(Dun) TCR contains 1 partial and 2 complete copies of the 68 bp P element and lacks the 39 bp Q and 63 bp R enhancer elements found in the archetype TCR. The BKV(AS) TCR contains one copy each of the P, Q and R elements. These sequences (P, Q and R) are represented by lines drawn below the BKV(WW) TCR structure and are read from left to right. A gap within these lines represents a deletion relative to the archetype sequence; parallel lines represent multiple copies of that region. Primers BK#2, BK#3, BK#4, BK#5 and BK#6 are represented by the numbers 2, 3, 4, 5 and 6 respectively, whereas BK(Arch1) and BK(Arch2) are shown as A1 and A2, respectively. Sequences encoding the early and late proteins are to the left of the BK#5, or right of the BK#4, annealing sites, respectively.

horseradish peroxidase conjugate (diluted 1:2000 in TBST) that would recognize anti-BKV or anti-SV40 antibodies immobilized in the wells. Once again the reaction between the primary (from serum) and the secondary (anti-IgG) antibodies was allowed to proceed for 2 hours at 37°C before excess anti-IgG was washed away with TBST. Bound horseradish peroxidase conjugate was detected by incubating the plates with 27.6 μM

o-phenylene-diamine (OPD) /H_2O_2 substrate in sodium citrate buffer (99.9 mM sodium citrate [pH 4.5]) for 5 minutes. The O_2 liberated from H_2O_2 dissociation oxidized the redox indicator *o*-phenylene-diamine and the resultant colorimetric change of OPD (from colorless to yellow) was measured at a wavelength of 450 nm in an ELISA plate reader (Dynex Labs, Chantilly, VA).

The anti-BKV antibody titer reported for each donor was determined from the inverse of the last dilution of the serum (endpoint) that yielded a spectrophotometric reading greater than twice the value obtained for the negative control tested in parallel. Two ELISA experiments were carried out using serum from each donor, and an antibody titer of 32 or greater was chosen arbitrarily as an indicator of the presence of relevant antibodies. Several controls were also included to ensure that the donors' sera did not react with uncoated (no antigen) ELISA plates and to examine the reactivity of known specific (anti-polyomavirus polyclonal antibodies) and non-specific (anti-T antigen monoclonal antibody, Pab 419) antibodies against the virions.

RESULTS

Detection of BKV DNA in PBLs

To detect the presence of BKV DNA in PBLs obtained from immunocompetent individuals, total cellular DNA was isolated from 200 μl of whole blood using the QiaAmp Blood Mini Kit. This method relies upon the initial lysis of erythrocytes followed by the extraction of DNA from PBLs that can then be used for PCR analysis. In control reactions all BKV primer pairs used in this study amplified 10 or fewer molecules of recombinant BKV DNA. Amplification was not observed with any of these primers when 1,000 copies of the SV40 or JCV genomes were used as target DNA (data not shown).

DNAs from all 40 donor blood specimens were tested by n-PCR using outer primer pair BK#4 and BK#5 and inner primer pair BK#2 and BK#3. Both sets of primers bind to conserved sequences that flank the hypervariable TCR. Because these primer pairs were designed to amplify both archetype and rearranged forms of BKV, our approach, in principle, would allow us to determine if both forms of BKV were present in PBLs (Fig. 1).

Positive PCR signals for the BKV TCR were detected in specimens from 22 of 40 (55%) donors. PCR products were cloned into the vector pCR™2.1 and sequenced (Fig. 2). The BKV TCRs amplified from all of the PBL DNA samples were found to represent the rearranged form of the virus and are predicted to have arisen by the duplication and deletion of sequences within an archetype TCR. The BKV TCR sequences amplified in this study differed from those of the BKV strains handled in our laboratory [BKV(Dun) and BKV(AS)], thereby arguing against the possibility of contamination of the donor DNAs by a "laboratory-strain" of BKV. One of the TCRs detected in this study, however, has been identified previously [Sundsfjord et al., 1990, 1994]. The TU strain of BKV was detected in the PBLs of 10 donors (1, 2, 8, 10, 12, 15, 19, 23, 38, 40). The TCR of this variant contains one intact copy of each of the P_{68} and Q_{39} enhancer elements and a partial copy of the R_{63} element containing the origin-proximal 12 base pairs (bps). Additionally, there exist partial duplications of 53 bps and 34 bps from the P_{68} and Q_{39} elements, respectively, adjacent to the origin-distal 12 bps from the R_{63} sequence (Fig. 2).

In addition to the TCR of the TU variant, unique BKV TCR sequences were amplified from the PBLs of 12 other donors (6, 7, 9, 13, 16, 17, 18, 20, 21, 30, 33, 39; Fig. 2), some of which seem to be related to BKV(TU). PBL DNAs from donors 9, 16 and 30 contained multiple rearranged forms of the TCR. Three variants, each having unique alterations within the P_{68}, Q_{39}, and R_{63} enhancer elements, were found in the specimen from donor 9. Similarly, two TCR variants were amplified from the DNA of each of donors 16 and 30 (Fig. 2). Sequence comparisons of the TCRs from these donors suggest that each arose independently from an archetype TCR and not from each other.

Identification of BKV DNA in PBLs Using Archetype-Specific Primers

Nested PCR conducted with primer pairs BK#4/BK#5 and BK#2/BK#3 has the potential to amplify both rearranged and archetype TCRs, however, our initial experiments only identified rearranged forms in the donors' PBLs. Because our approach of cloning and sequencing PCR products favored the detection of a predominant form, it was possible that an archetype variant might be present, but at a low level relative to the rearranged variants [Newman and Frisque, 1997]. To test this hypothesis, archetype-specific primers, BK(Arch1) and BK(Arch2), were designed. These primers anneal to a sequence within the R_{63} enhancer element that is present in the archetype TCR but not as a single intact block in any of the rearranged TCRs (Fig. 1). When primers BK(Arch1) and BK(Arch2) were used in combination with primers BK#3 and BK#6, respectively, the archetype TCR was amplified from 5 of the 6 BKV-positive donors tested (Fig. 3). This archetype sequence is identical to that of the WW archetype strain [Rubinstein et al., 1987; Negrini et al., 1991] except at nucleotide positions 52 and 65 ["A" and "T" in BKV(WW) and "T" and "C" in BKV(TU), BKV(Dun) and in all TCRs amplified in this study].

Amplification from donor DNA specimens of an archetype TCR containing a P_{68} element and a "G", "A," and "G" nucleotide at position 89, 147, and 182, respectively, eliminated the possibility that the amplified DNA represented a carryover contamination with BKV(AS), the only BKV strain in our laboratory having an archetype-like TCR. In addition, all rearranged TCRs amplified in this study had the same nucleotide changes at polymorphic sites 52, 65, 89, 147, and 182 as the amplified archetype TCR. These findings are consistent with the prediction that the amplified rearranged TCRs (Fig. 2) emerged from the archetype TCR found in PBL DNA by the process of deletion and duplication (Fig. 3).

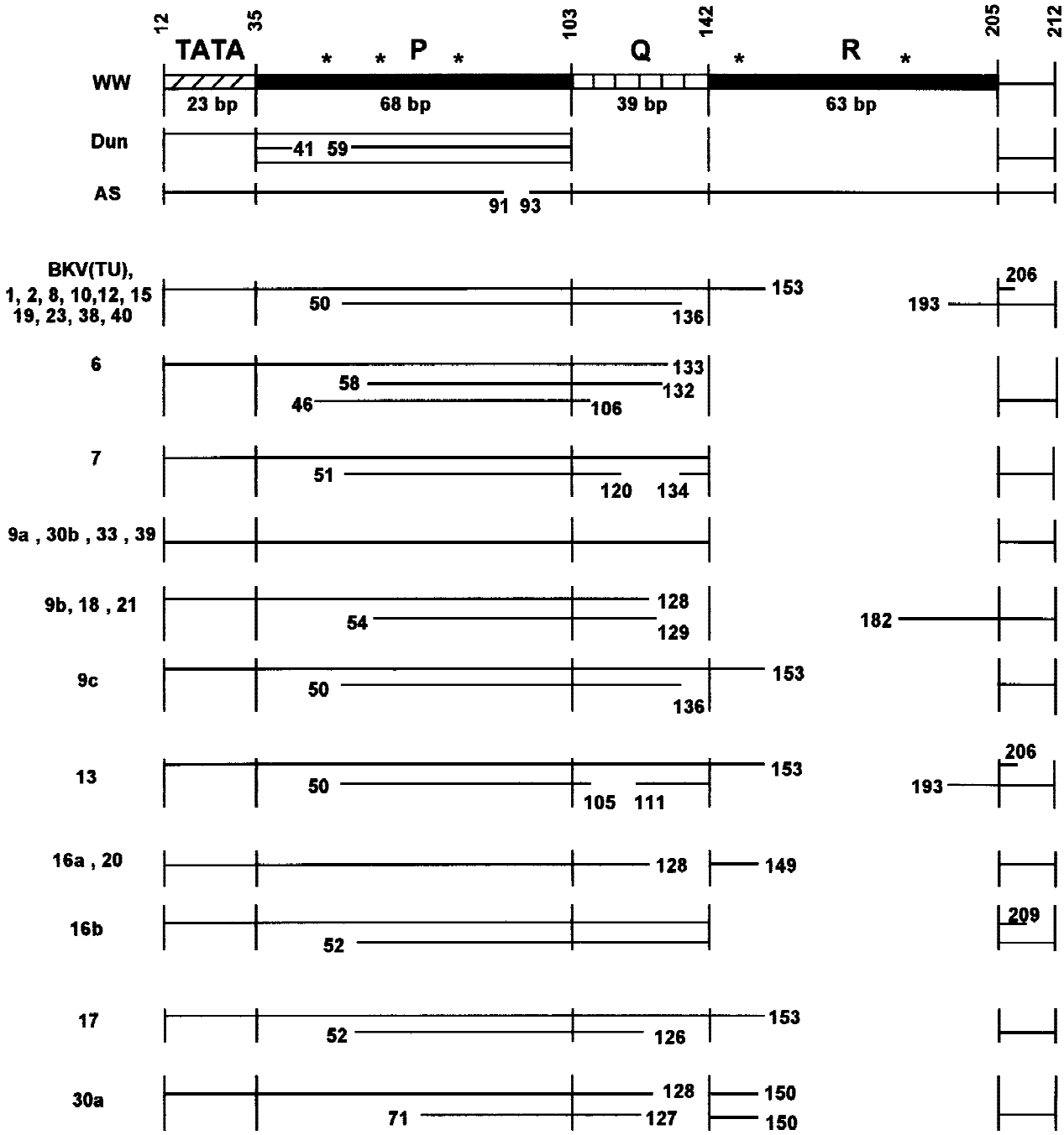

Fig. 2. Structures of BKV TCRs amplified from PBLs of healthy donors. BKV TCR sequences amplified from donor leukocyte DNA (identified on the left by their assigned number of 1–40) are compared to each other and to the TCRs of three known strains of BKV: WW, Dun and AS. The BKV(WW) TCR (archetype) is shown at the top of the figure and serves as the reference sequence to which the other TCRs are compared. The numbers across the top represent nucleotide positions using BKV(WW) numbering [Rubinstein et al., 1987]. The BKV(WW) TCR contains enhancer elements P (68 bp), Q (39 bp) and R (63 bp) and a 23 bp sequence that contains the TATA box. The P, Q and R sequences are represented as described in the legend to Figure 1. The numbers at the boundaries of gaps and duplications represent the nucleotide positions of the fragment deleted or duplicated with respect to the BKV(WW) strain. TCR sequences amplified from 10 donors were identical to that of BKV(TU); TCR sequences from other donors were unique. Structures 9a, b, c, 16a ,b and 30a, b represent the multiple TCR variants amplified from donors 9, 16 and 30, respectively. Asterisks within the P and R elements denote nucleotide polymorphisms that occur at positions 52, 65, 89, 147 and 182 in different BKV isolates. BKV(TU) and all BKV TCRs amplified in this study differ from BKV(WW) at nucleotides 52 and 65 and from BKV(AS) at nucleotide 89. Additionally, those TCRs structures that retain nucleotides 147 or 182 differ from BKV(AS) at these positions.

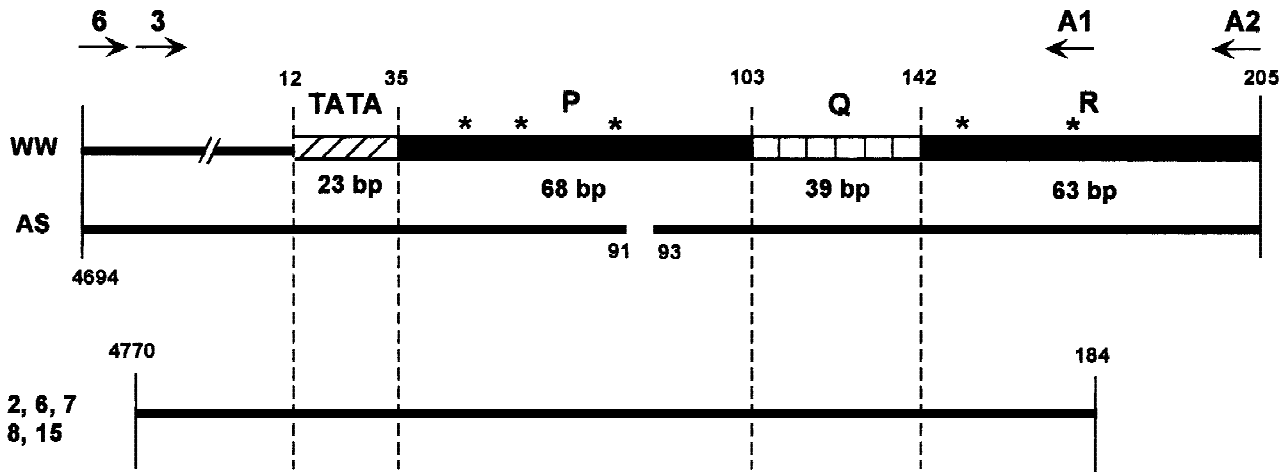

Fig. 3. Structures of BKV TCRs amplified from donors' PBLs using archetype-specific primers. The structures of the BKV(WW) and BKV(AS) TCRs are as described in the legend to Figure 1. The left most boundary shown is at nucleotide position 4694 [BKV(AS) numbering; Tavis et al., 1989]. Archetype BKV TCR sequences amplified from donors' PBLs are represented by the line shown below the BKV(WW) and BKV(AS) TCRs. Archetype BKV TCR sequences were amplified from PBLs of donors 2, 6, 7, 8 and 15 using n-PCR. Primers BK#3, BK#6, BK(Arch1) and BK(Arch2) are represented as 3, 6, A1 and A2, respectively. Oligonucleotide pairs BK#6/BK(Arch2) and BK#3/BK(Arch1) were used as outer and inner primer sets, respectively. Asterisks within the P and R elements denote the 5 nucleotide polymorphisms discussed in the legend to Figure 2. The archetype sequence is identical to the BKV(TU) sequence at the polymorphic positions 52, 65, 89 and 147; at position 182, that is missing in BKV(TU), the nucleotide in the archetype sequence is a 'G' as in BKV(WW).

Detection of Anti-BKV Antibodies in Serum

Serological and PCR analyses both indicate that BKV establishes a persistent infection in a high percentage of individuals; however, a direct correlation between the detection of anti-viral antibodies and viral DNA sequences has not been examined in a group of immunocompetent individuals. To confirm that donors who were BKV-positive by the PCR approach also produced antibodies to the virus, an ELISA was developed.

Sera from 21 of the 22 donors demonstrated to be BKV- positive by PCR had anti-BKV titers $\geq$32 (range 32–4096); 1 donor (33) who was PCR-positive had a borderline anti-BKV antibody titer in 1 of 2 experiments (Table II). Seventeen of 18 PCR-negative donors also had anti-BKV titers. A total of 38 of 40 donors (95%) were considered to be seropositive for BKV (titer $\geq$32) (Table II).

Detection of Anti-SV40 Antibodies in Serum

The availability of the 40 donor sera permitted us to address the possibility that humans are exposed routinely to SV40. Sera from 3 donors (5, 7 and 8) reacted with the SV40(776) virions used in the ELISA (Table II). Two of these sera, #7 and #8, had only borderline activity (anti-SV40 titer of 32), whereas serum #5 had a significant titer of 512 in both ELISA experiments. The measurement of high anti-BKV titers in the absence of significant anti-SV40 titers in several of the sera suggests that serum #5 did, in fact, contain specific anti-SV40 antibodies rather than anti-BKV antibodies that simply cross-reacted with the SV40 antigen.

DISCUSSION

Exposure of the human population to BKV is nearly universal [Gardner, 1973; Brown et al., 1975]. Results from a number of BKV and JCV studies indicate that the archetype form of each virus establishes primary infections in children and adolescents, persists in kidney tissue, and is shed in the urine. Rearranged forms of BKV, that are thought to arise from archetype virus, have been detected in urine, suggesting that these variants may be transmitted between individuals as well [Heritage et al., 1981; Apperley et al., 1987]. Archetype and rearranged forms may exhibit overlapping tissuetropic properties that determine their abilities to establish infections at distinct secondary sites after their hematogenous spread from a primary site. Questions regarding this spread of virus within the healthy host remain to be answered. Whereas Dörries and coworkers [1994] did detect BKV DNA in PBLs and suggested that these cells may transport the virus throughout the body, the number of leukocyte specimens analyzed were limited and the predominant form of BKV detected was related to BKV(Dun), a common laboratory strain.

We have also employed the highly sensitive and specific PCR technique to detect and analyze BKV DNA in PBLs. Evidence was obtained that indicated that BKV DNA was present in 22 of the 40 (55%) donor specimens. By comparing the intensity of PCR signals obtained from donor DNA samples with those of positive controls, we concluded that BKV is present in the donors' PBLs at very low levels. This observation might indicate that false-negative reactions occurred in some of the remaining 18 samples; BKV DNA may have been present, but because of very low copy numbers or the

TABLE II. Summary of Results From ELISA and PCR Analyses of 40 Donor Specimens

Specimen[a]	Detection method	#BKV$^+$/Total	(%)
PBLs (all donors)	PCR	22/40	(55)
Serum (all donors)	ELISA	38/40[b]	(95)
PBLs, ELISA$^+$ donors[c]	PCR	21/38	(55)
PBLs, ELISA$^-$ donors	PCR	1/2	(50)
Serum, PCR$^+$ donors	ELISA	21/22	(95)
Serum, PCR$^-$ donors	ELISA	17/18	(94)

[a]PBL and serum specimens were obtained from each of the 40 donors and analyzed for BKV DNA by PCR and anti-BKV antibodies by ELISA, respectively.
[b]An antibody titer of 32 or greater in both ELISA was considered indicative of the presence of anti-BKV antibodies in a serum sample. Three sera (7.5%) contained anti-SV40 antibodies; only 1 (2.5%) of these had a titer >32.
[c]PBL specimens obtained from donors known to contain anti-BKV antibodies in their sera, as determined by ELISA, were analyzed for BKV DNA by PCR.

presence of PCR inhibitors in the samples [Morata et al., 1998], it went undetected. The possibility that inhibitors did affect the limits of detection of the assay is supported by our finding that, in some instances, amplification of the endogenous CD19 control sequence and the BKV DNA sequences required dilution of the PBL DNA specimen in sterile water (data not shown). Dilution of the 18 PBL DNA samples that were initially BKV-negative, however, did not yield any new BKV-positive specimens.

Several unique forms of the BKV TCR were detected in the donor population. Rearranged BKV TCRs amplified from PBLs contained deletions of most or all of the R_{63} enhancer sequence. Grinnell and coworkers [1988] reported that origin-proximal sequences within the R_{63} element negatively regulate the BKV enhancer. In addition, Sugimoto's group [1989] found that archetype BKV(WW), that includes the R_{63} element in its TCR, grew inefficiently in cell culture relative to rearranged BKV(PT) that lacks an R_{63} sequence in its TCR . It is possible that naturally-occurring BKV variant genomes that have deleted a "silencer" element within the R_{63} sequence (such as those identified in Fig. 2), have a replicative advantage in leukocytes because their TCRs are transcriptionally more active than that of the archetype variant in these cells.

The BKV(TU) TCR was identified in 10 of 22 (45%) BKV-positive samples. BKV(TU) was originally detected, along with archetype-like BKV(WWT), in urine samples of HIV-positive individuals, pediatric cancer patients and healthy control subjects [Sundsfjord et al., 1990; Flægstad et al.,1991]. Other reports have identified archetype BKV(WW) as the predominant variant detected in urine [Mew et al., 1981; Chauhan et al., 1984; Markowitz et al., 1991]. The presence of BKV(WWT) and BKV(TU) in the urine of healthy people indicates that archetype BKV(WW) is not the only form of the virus circulating in the population. These two variants may differ in their ability to multiply in kidney tissue and to infect susceptible individuals. Because BKV(WWT) was the predominant form in the urine samples, this strain may have a replicative advantage in the kidneys; however, when a mixed inoculum containing both BKV(WWT) and BKV(TU) was passaged in human embryonic kidney cells, the latter strain outgrew the former [Sundsfjord et al., 1990], suggesting that the BKV(TU) TCR confers a replicative advantage upon the virus in cell culture.

The tonsils have been proposed to be a primary site of infection for the human polyomaviruses based upon the detection of BKV and JCV in this tissue and upon the ability of JCV to replicate in cultures of tonsillar stromal cells [Goudsmit et al., 1982; Monaco et al., 1996, 1998]. The detection of the same rearranged form, BKV(TU), in leukocytes (this study) and in urine [Sundsfjord et al., 1990], strengthens the hypothesis that lymphocyte-mediated transport of rearranged BKV variants occurs from a primary site of infection (e.g., tonsils) to secondary sites (e.g., kidney) within the body. The observation that circulating B-lymphocytes are in close association with the tonsillar stroma [Lisignoli et al., 1996] suggests a mechanism by which human polyomaviruses may leave the primary site to enter the circulation.

Donor sera obtained in the present study were assayed for anti-BKV antibodies using ELISA to confirm the expected correlation between the presence of viral DNA in leukocytes and BKV seropositivity. Initial optimization of the ELISA protocol was carried out using sera that had been titered by HAI. Compared to the latter technique, ELISA was found to be more sensitive (both at high and low concentrations of anti-BKV antibodies) and more rapid to employ. An antibody titer of 32 or greater was considered to be indicative of the presence of specific antibodies in serum. By this criterion, 38 of 40 (95%) serum samples contained anti-BKV antibodies (Table II). Most individuals found to have BKV DNA in their PBLs also harbored anti-BKV antibodies in their sera, although one donor who was PCR-positive had only a borderline anti-BKV antibody titer. BKV DNA was not detected in the blood of many people who were serologically positive for the virus. It is possible that BKV is not in the PBLs of all individuals exposed to BKV, or that it is present at levels too low to be detected by our approach.

Recent PCR experiments suggest that SV40, a contaminant in the poliovirus vaccine administered to millions of people between 1955 and 1963 [Shah and Nathanson, 1976; Strickler et al., 1998], is present in several types of human tumors and may circulate in

the human population [Carbone et al., 1994; Lednicky et al., 1995; Carbone et al., 1996; Martini et al., 1996; Carbone et al., 1997]. If true, we might have expected to readily detect anti-SV40 antibodies in our donors' sera, however, high titers of such antibodies were observed in only 1 of the 40 (2.5%) specimens. This value is in close agreement with that of several previous reports (5%) [reviewed in Shah 1998], and it is lower than that published in a recent report by Jafar et al., (~12%) [1998]. These minor discrepancies in percent seropositivity are likely due to differences in the serologic assay employed and in the titer chosen to denote a specific antibody response. Taking into account the PCR results from other laboratories, our serological findings could be interpreted in at least three ways: i) SV40 may circulate in the population, but only inefficiently, ii) the SV40 (776) strain used as antigen in the ELISA may be antigenically distinct from most SV40 variants infecting humans or iii) SV40 does not circulate in humans and the rare positive serological reaction is a result of an accidental SV40 exposure or a cross-reaction with an as yet unidentified human polyomavirus.

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REFERENCES

Agostini HT, Brubaker GR, Shao J, Levin A, Ryschkewitsch CF, Blattner WA, Stoner GL. 1995. BK virus and a new type of JC virus excreted by HIV-1 positive patients in rural Tanzania. Arch Virol 140:1919–1934.

Apperley JF, Rice SJ, Bishop JA, Chia YC, Krauz T, Gardner SD, Goldman JM. 1987. Late onset hemorrhagic cystitis associated with urinary excretion of polyomavirus after bone marrow transplantation. Transplantation 43:108–112.

Arthur RR, Shah KV, Baust SJ, Santos GW, Saral R. 1986. Association of BK viruria with hemorrhagic cystitis in recipients of bone marrow transplants. N Engl J Med 315:230–234.

Arthur RR, Shah KV. 1989. Occurrence and significance of papovavirus BK and JC in the urine. Prog Med Virol 36:42–61.

Ault GS, Stoner GL. 1993. Human polyomavirus JC promoter/enhancer rearrangement patterns from progressive multifocal leukoencephalopathy brain are unique derivatives of a single archetypal structure. J Gen Virol 74:1499–1507.

Brown P, Tsai T, Gajdusek GD. 1975. Seroepidemiology of human polyomavirus. Discovery of virgin populations and some unusual pattern of antibody prevalence among remote people of the world. Am J Epidemiol 102:331–340.

Caputo A, Corallini A, Grossi MP, Carra L, Balboni PG, Negrini M, Milanesi G, Federspil, Barbanti-Brodano G. 1983. Episomal DNA of a BK virus variant in human insulinoma. J Med Virol 12:37–49.

Carbone M, Pass HI, Rizzo P, Marinetti M, Di Muzio M, Mew DJY, Levin AS, Propocio A. 1994. Simian virus-40 like DNA sequences in human pleural mesothelioma. Oncogene 9:1781–1790.

Carbone M, Rizzo P, Propocio A, Guiliano M, Pass HI, Gebhardt MC, Mangham C, Hansen M, Malkin DF, Bushart G, Pompetti F, Picci P, Levine AS, Bergsagel JD, Garcea RL. 1996. SV-40 like sequences in human bone tumors. Oncogene 13:527–535.

Carbone M, Rizzo P, Pass HI. 1997. Simian virus 40, polio vaccines and human tumors: a review of the recent developments. Oncogene 15:1877–1888.

Chauhan S, Lecatsas G, Harley EH. 1984. Genome analysis of BK(WW) viral DNA cloned directly from human urine. Intervirology 22:170–176.

Chesters PM, Heritage J, McCance DJ. 1983. Persistence of DNA sequences of BK virus and JC virus in normal human tissues and in diseased tissues. J Infect Dis 147:676–684.

Coleman DE, Mackenzie EFD, Gardner SD, Poulding JM, Amer B, Russel WJI. 1978. Human polyomavirus BK infection and ureteric stenosis in renal allograft recipients. J Clin Pathol 31:338–347.

Corallini A, Pagnani M, Viadana P, Silini E, Mottes M, Milanesi G, Gerna G, Vettor R, Trapella G, Silvani V, Gaist G, Barbanti- Brodano G. 1987. Association of BK virus with human brain tumors and tumors of pancreatic islets. Int J Cancer 39:60–67.

Dei R, Marmo F, Corte D, Sampietro MG, Franceschini E, Urbano P. 1982. Age-related changes in the prevalence of precipitating antibodies to BK virus in infants and children. J Med Microbiol 15: 285–291.

Dörries K, Loeber G, Meixensberger J. 1987. Association of polyomavirus JC, SV40 and BK with human brain tumors. Virology 62: 3378–3387.

Dörries K, Vogel E, Gunther S, Czub S. 1994. Infection of human polyomavirus JC and BK in peripheral blood leukocytes from immunocompetent individuals. Virology 198:50–70.

Flægstad T, Traavik T, Kolmannskog S, Stokland T. 1988. BK virus infection in children with cancer: serological response studied by hemagglutination inhibition, neutralization and IgG and IgM-class specific ELISA tests. J Med Virol 24:33–44.

Flægstad T, Sundsjford RR, Arthur M, Perderson T, Traavik T, Subramani S. 1991. Amplification and sequencing of control regions of JC and BK virus from human urine by polymerase chain reaction. Virology 180:553–560.

Gardner SD, Field AM, Coleman DV, Hulme B. 1971. New human papovavirus BK isolated from urine after renal transplantation. Lancet I:1253–1257.

Gardner SD. 1973. Prevalence in England of antibodies to human polyomavirus BK. Br Med J 1:77–78.

Gardner SD, Mackenzie EFD, Smith C, Porter AA. 1984. Prospective study of the human polyomavirus BK and JC and cytomegalovirus in renal transplant recipients. J Clin Pathol 37:578–586.

Goudsmit JP, Wertheim-van Dillan, van Strein A, van der Noordaa A. 1982. The role of BK virus in acute respiratory tract diseases and the presence of BKV DNA in tonsils. J Med Virol 10:91–99.

Grinnell BW, Berg DT, Walls JD. 1988. Negative regulation of the human polyomavirus BK enhancer involves cell specific interaction with a nuclear repressor. Mol Cell Biol 8:3448–3457.

Heritage J, Chesters PM, McCance DJ. 1981. The persistence of papovavirus BK DNA sequences in normal human renal tissue. J Med Virol 8:143–150.

Houff SD, Major EO. 1989. JCV infected cells in the spleen and bone marrow are involved in the pathogenesis of progressive multifocal leukoencephalopathy. N Engl J Med 318:301–305.

Jackson DP, Hayden JD, Quirke P. 1991. Extraction of nucleic acid from fresh and archival material. In: McPherson MJ, Quirke P, Taylor GR, editors. PCR: a practical approach. New York: Oxford University Press. p 29–50.

Jafar S, Rodriguez-Barradas M, Graham DY, Butel JS. 1998. Serological evidence of SV40 infections in HIV-infected and HIV-negative adults. J Med Virol 54:276–284.

Jin L, Gibson PE, Knowles WA, Clewley JP. 1993. BK virus antigenic variants: sequence analysis within the capsid VP1 epitope. J Med Virol 39:50–56.

Jin L, Pietropaolo V, Booth JC, Ward K, Brown DW. 1995. Prevalence and subtype distribution of BK virus in healthy people and immunocompromised patients detected by PCR-restriction enzyme analysis. Clin Diag Virol 3:285–295.

Knowles WA, Gibson PE, Gardner SD. 1989. Serological typing scheme for BK-like isolates of human polyomavirus. J Med Virol 28:118–123.

Kwok S. 1990. Procedures to minimize PCR-product carryover. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR protocols. A guide to methods and applications. San Diego: Academic Press, Inc. p 142–145.

Lednicky JA, Garcea RL, Bergsagel DJ, Butel JS. 1995. Natural simian virus 40 strains present in human choroid plexus and ependymoma tumors. Virology 212:710–717.

Lisignoli G, Monaco MCG, Degrassi A. 1996. In vitro cultured cells from human tonsils display a distinct phenotype and induce B-cell adhesion and proliferation. Eur J Immunol 26:17–27.

Loeber G, Dörries K. 1988. DNA rearrangement in organ-specific variants of polyomavirus JC strain GS. J Virol 62:1730–1735.

Markowitz RB, Eaton BA, Kubik MF, Latorra D, McGregor JA, Dynan WS. 1991. BK virus and JC virus shed during pregnancy have predominantly archetypal regulatory regions. J Virol 65:4515–4519.

Martin JD, King DM, Slauch JM, Frisque RJ. 1985. Differences in regulatory sequences in naturally occurring JC virus variants. J Virol 53:306–311.

Martini F, Iaccheri L, Lazzarin L, Carinci P, Corallini A, Gerosa M, Iuzzolino P, Barbanti-Brodano G, Tognon M. 1996. SV-40 early region and large T antigen in human tumors, peripheral blood cells and sperm fluids from healthy individuals. Cancer Res 56: 4820–4825.

Mathurm US, Olson JL, Darragh TM, Yen TJB. 1997. Polyomavirus induced interstitial nephritis in two renal transplant recipients: case reports and review of the literature. Am J Kidney Dis 29:754–758.

McCance DJ, Gardner SD. 1987. Papovaviruses: papillomaviruses and polyomavirus. In: Zuckerman AJ, Banatwala JE, Pattison JR, editors. Principles and practice of clinical virology. Chichester: John Wiley Press. p 479–506.

Mew RT, Lecatsas TG, Prozesky OW, Harley EH. 1981. Characteristics of BK papovavirus DNA prepared directly from human urine. Intervirology 16:14–19.

Monaco MCG, Atwood WJ, Major EO. 1996. JC virus infection of hematopoietic progenitor cells, primary B-lymphocytes and tonsillar stromal cells. J Virol 70:7004–7012.

Monaco MCG, Jensen PN, Hou J, Durham LC, Major EO. 1998. Detection of JC virus DNA in human tonsil tissue: evidence for site of initial viral infection. J Virol 72:9918–9923.

Morata P, Quiepo-Ortuno MI, de Dios Colmenero J. 1998. Strategy for optimizing DNA amplification in a peripheral blood PCR assay used for diagnosis of human brucellosis. J Clin Microbiol 36:2443–2446.

Newman JT, Frisque RJ. 1997. Detection of archetype and rearranged variants of JC virus in multiple tissues from a pediatric PML patient. J Med Virol 53:243–252.

Newman JT, Frisque RJ. 1999. Identification of JC virus variants in multiple tissues of pediatric and adult PML patients. J Med Virol 58:79–86.

Negrini M, Sabbioni S, Arthur RR, Castagnoli A, Barbanti-Brodano G. 1991. Prevalence of the archetypal regulatory region and sequence polymorphisms in nonpassaged BK virus variants. J Virol 65:5092–5095.

Prince AM, Andrus L. 1992. PCR-How to kill unwanted DNA. Biotecniques 12:358–360.

Rubinstein R, Pare N, Harley EH. 1987. Structure and function of the transcriptional control region of nonpassaged BK virus. J Virol 61:1747–1750.

Seif I, Khoury G, Dhar R. 1979. The genome of human papovavirus BKV. Cell 18:963–977.

Shah KV. 1998. SV40 infections in simians and humans. In: Brown F, Lewis AM, editors. Simian virus 40 (SV40): a possible human polyomavirus. Basel: Kargel. p 9–12.

Shah K, Nathanson N. 1976. Human exposure to SV40: review and comment. Am J Epid 103:1–12.

Shah KV, Daniel RW, Warszavski RM. 1973. High prevalence of antibodies to BK virus, an SV40-related papovavirus in residents of Maryland. J Infect Dis 128:784–787.

Smith RD, Galla JH, Skahan K, Anderson P, Linnemann Jr. CC, Ault GS, Ryschkewitsch CF, Stoner GL. 1998. Tubulointerstitial nephritis due to a mutant polyomavirus BK virus strain, BKV(Cin), causing end-stage renal disease. J Clin Microbiol 36:1660–1665.

Stamenkovic I, Seed B. 1988. CD19, the earliest differentiation antigen of the B-cell lineage, bears the extracellular immunoglobulin-like domains and an Epstein-Barr virus-related cytoplasmic tail. J Exp Med 168:1205–1210.

Strickler HD, Rosenberg PS, Devesa SS, Hertel J, Fraumeni JF, Goebert JJ. 1998. Contamination of poliovirus vaccine with simian virus 40 (1955–1963) and subsequent cancer rates in the United States. JAMA 279:292–295.

Sugimoto C, Hara K, Taguchi F, Yogo Y. 1989. Growth efficiency of naturally occurring BK virus variants in vivo and in vitro. J Virol 63:3195–3199.

Sundsjford A, Johansen T, Flægstad T, Moens U, Villand P, Subramani S, Traavik T. 1990. At least two types of control regions can be found amongst naturally occurring BK virus strains. J Virol 64:3864–3871.

Sundsjford A, Flægstad T, Flø R, Spein AR, Pederson M, Permin H, Julsrad J, Traavik T. 1994. BK and JC viruses in human immunodeficiency virus type-1 infected persons: prevalence, excretion, viremia and viral regulatory regions. J Inf Dis 169:485–490.

Takemoto KK, Rabson AS, Mullarky MF, Blaese RM, Garon CF, Nelson D. 1974. Isolation of papovavirus from brain tumor and urine of a patient with Wiskott-Aldrich syndrome. J Natl Cancer Inst 53:1205–1207.

Tavis JE, Walker DL, Gardner SD, Frisque RJ. 1989. Nucleotide sequence of human polyomavirus AS virus, an antigenic variant of BK virus. J Virol 63:901–911.

Tedder TF, Isaacs CM. 1989. Isolation of cDNA encoding the CD19 antigen of the human and mouse B-lymphocytes. J Immunol 143: 712–717.

Tominaga T, Yogo Y, Kitamura T, Aso Y. 1992. Persistence of archetypal JC virus DNA in normal renal tissues derived from tumor bearing patients. Virology 186:736–741.

White III FA, Ishaq M, Stoner GL, Frisque RJ. 1992. JC virus DNA is present in many human brain samples in patients without progressive multifocal leukoencephalopathy. J Virol 66:5726–5734.

Yogo Y, Kitamura T, Sugimoto C, Hara K, Iida T, Taguchi F, Tajima A, Kawabe K, Aso Y. 1991. Sequence rearrangement in JC virus molecularly cloned from immunosuppressed renal transplant patients. J Virol 65:2422–2428.

Yogo Y, Kitamura T, Sugimoto C, Ueki T, Aso Y, Hara K, Taguchi F. 1990. Isolation of a possible archetypical JC virus DNA sequence from nonimmuno-compromised individuals. J Virol 64:3139–3143.